
PARAPHYLY OF *IXORA* AND NEW TRIBAL DELIMITATION OF IXOREAE (RUBIACEAE): INFERENCE FROM COMBINED CHLOROPLAST (*RPS16*, *RBCL*, AND *TRNT-F*) SEQUENCE DATA¹

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ABSTRACT

We performed phylogenetic analyses of DNA sequences of three chloroplast markers: *rbcL*, *rps16*, and *trnT-F*, to rigorously test the monophyly of competing circumscriptions of the tribe Ixoreae. Several genera traditionally or currently associated with the type genus *Ixora* L. were included in the analyses. *Ixora* as currently circumscribed appears paraphyletic, as many other genera are nested within it with strong support: *Captaincookia* N. Hallé, *Doricera* Verdc., *Hitoa* Nadeaud, *Myonima* Comm. ex Juss., *Sideroxyloides* Jacq., *Thouarsiora* Homolle ex Arènes, and *Versteegia* Valetton. Further, *Aleisanthia* Ridl., *Aleisanthiopsis* Tange, and *Greenea* Wight & Arn. are more closely related to *Ixora* and allies than the monotypic genus *Scyphiphora* C. F. Gaertn. Consequently, Ixoreae fide Andreassen and Bremer (2000) is not monophyletic without an exclusion of *Scyphiphora*. Ixoreae fide Robbrecht and Manen (2006) is not monophyletic unless *Captaincookia* and *Doricera* are included. The monophyly of a morphologically heterogeneous Ixoreae alliance consisting of *Ixora* and its relatives *Aleisanthia*, *Aleisanthiopsis*, and *Greenea* is, however, strongly supported. In order to recognize monophyletic and morphologically consistent groups, we adopt a narrow circumscription of Ixoreae (including *Bemsetia* Raf., *Captaincookia*, *Charpentiera* Vieill., *Doricera*, *Hitoa*, *Ixora*, *Myonima*, *Pancheria* Montrouz., *Sideroxyloides*, *Thouarsiora*, *Tsiangia* But, H. H. Hsue & P. T. Li, and *Versteegia*), and two new tribes are erected for *Aleisanthia* + *Aleisanthiopsis* and *Greenea*, respectively. The Indo-Malesian Aleisanthieae and the pantropical Ixoreae s. str. are sister groups, and the Southeast Asian Greeneeae is sister to the Ixoreae–Aleisanthieae clade.

Key words: Aleisanthieae, cpDNA, Greeneeae, *Ixora*, Ixoreae, Ixoroideae, phylogeny, Rubiaceae.

The large pantropical genus *Ixora* L. was earlier classified either in the tribe Pavetteae (Richard, 1829; Dumortier, 1829) or the tribe Coffeae (Wight & Arnott, 1834) until Gray (1858) placed it in his new tribe Ixoreae (Table 1), which now belongs to the subfamily Ixoroideae sensu Bremer et al. (1999). Ixoreae initially consisted of two large Linnaean genera, *Ixora* (the type genus) and *Pavetta* L., with contorted aestivation and peltate or centrally affixed ovules (Gray, 1858), but the genus *Coffea* L., presently classified in Coffeae, was later added (Hooker, 1873; Bremekamp, 1934). From 1979 to 2000, both *Ixora*

and *Pavetta* were placed either in Coffeae (Darwin, 1979; Verdcourt, 1989) or in Pavetteae (Bridson & Robbrecht, 1985; Robbrecht, 1988). Phylogenetic studies of Ixoroideae conducted by Andreassen and Bremer (1996, 2000) based on the combined molecular and morphological data demonstrated for the first time that *Ixora* and *Pavetta* were not closely related genera. The monotypic and mangrove genus *Scyphiphora* C. F. Gaertn. (Gaertner, 1806), formerly classified in the tribe Gardenieae sensu Robbrecht (1988), was resolved with moderate support (jackknife [JK] – 70%, Andreassen & Bremer, 2000: 1740) as

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sister to a strongly supported (JK — 100%, Andreasen & Bremer, 2000: 1740) clade consisting of four species of *Ixora* and one species each of the genera *Myonima* Comm. ex Juss. (Jussieu, 1789) and *Versteegia* Valetton (Valeton, 1911). Accordingly, Andreasen and Bremer (2000) reinstated and recircumscribed Ixoreae to include *Captaincookia* N. Hallé (Hallé, 1973), *Doricera* Verdc. (Verdcourt, 1983, 1989), *Ixora*, *Myonima*, and *Versteegia*; they tentatively placed *Scyphiphora* there. The tribe comprises ca. 510 species, some of which are economically important (e.g., the ornamental *I. coccinea* L., *I. finlaysoniana* Wall. ex G. Don, and *I. hookeri* (Oudem.) Bremek.). It is characterized by a combination of articulated petioles (De Block, 1998), terminal inflorescences, and 4-merous flowers with aestivation contorted to the left (Andreasen & Bremer, 2000). Pavetteae and Coffeeae were recircumscribed in a narrow sense.

Andreasen and Bremer (2000) also revealed that *Ixora*, represented by four species, was paraphyletic or polyphyletic. About 500 species (98%) of Ixoreae were encountered in the pantropical *Ixora* (Mouly, 2007). Several described monotypic genera, including *Becheria* Ridl. (Ridley, 1912), *Bemsetia* Raf. (Rafinesque, 1838), *Hitoea* Nadeaud (Nadeaud, 1899), *Pancheria* Montrouz. (Montrouzier, 1860; nom. rej.), *Patabea* Aubl. (Aublet, 1775), *Sideroxyloides* Jacq. (Jacquin, 1763), and *Thouarsiora* Homolle ex Arènes (Arènes, 1960), are considered to fall within *Ixora* based on morphology (Bentham, 1850; Beauvisage, 1901; Fosberg, 1937; Sandwith, 1937; Guédès, 1986; Smith & Darwin, 1988; De Block, 1998). The monotypic genus *Charpentiera* Vieill. (nom. illeg.), previously attributed to the family Loganiaceae (Vieillard, 1865), was included in *Ixora* (Rubiaceae) by Beauvisage (1901). The genus *Tsiangia* But, H. H. Hsue & P. T. Li (But et al., 1986) was described for a species based on a single specimen from Hong Kong, but Bridson (2000) stressed that this species was a deviant parasitized collection of *I. chinensis* Lam. *Captaincookia*, *Doricera*, and *Scyphiphora* remain monotypic genera, and *Myonima* and *Versteegia* contain only four and five species, respectively. Baillon (1879) merged *Myonima* with *Ixora* as a section, but subsequent Rubiaceae taxonomists did not follow this.

A recent phylogenetic study by Rova et al. (2002: 149) based on the *trnL-F* chloroplast region revealed for the first time a close relationship of *Ixora* with three Southeast Asian genera, *Aleisanthia* Ridl. (Ridley, 1920; Tange, 1996a), *Aleisanthiopsis* Tange (Tange, 1996b), and *Greenea* Wight & Arn. (Wight & Arnott, 1834), which were formerly classified in the tribe Rondeletieae of the subfamily Cinchonoideae

(Robbrecht, 1988). Malesian *Aleisanthia* and Indonesian *Aleisanthiopsis* are two small genera, each with two species. *Greenea* is a genus from Southeast Asia (Puff et al., 2005) with ca. 10 species. These three rainforest genera have contorted aestivation, scorpioid inflorescences, and capsular multiseeded fruits, features unknown in Ixoreae sensu Andreasen and Bremer (2000). More recently, Robbrecht and Manen (2006) proposed a broad circumscription of Ixoreae including *Ixora*, *Myonima*, *Scyphiphora*, *Versteegia*, and the above three genera. Their Ixoreae is diagnosed by contorted corolla aestivation, a feature commonly found in Ixoroideae s.l.

Sequencing data from the *rps16*, *trnT-F*, and *rbcL* chloroplast markers have been used separately and/or in combination with that of the ITS of nuclear ribosomal DNA (nrDNA) for assessing phylogenetic relationships in some rubiaceous groups (e.g., Razafimandimbison & Bremer [2001] for *rbcL* and ITS; Razafimandimbison & Bremer [2002], Lantz & Bremer [2004], Alejandro et al. [2005] for *trnT-F* and ITS). Here, we perform phylogenetic analyses using DNA sequencing data from the three chloroplast regions to reconstruct a robust phylogeny for Ixoreae. The resulting phylogeny from the combined data is used to rigorously test the monophyly of previous circumscriptions.

MATERIAL AND METHODS

TAXONOMIC SAMPLING

We used 33 species (Appendix 1) comprising seven *Ixora* species with a representative geographical range, one individual each for the monotypic genera *Captaincookia*, *Doricera*, *Hitoea*, *Scyphiphora*, *Sideroxyloides*, and *Thouarsiora*, a specimen of one species each for *Myonima*, *Versteegia*, *Aleisanthia*, and *Aleisanthiopsis* (two accessions), five representatives of *Greenea*, two members of the tribe Vanguerieae (*Peponidium cystiporon* (Byn. ex Cavaco) Razafim., Lantz & B. Bremer and *Cyclophyllum deplanchei* Hook. f.), and one species each for the nine formally recognized tribes of Ixoroideae s.l. (Andreasen & Bremer, 2000; Rova et al., 2002), notably *Pavetta* for Pavetteae. Despite available names under *Ixora*, the species of the genera *Hitoea* and *Sideroxyloides* are used in the study to clearly assess the circumscription of Ixoreae, with those of *Ixora* being addressed in another study. *Luculia gratissima* (Wall.) Sweet was used as outgroup taxon (Appendix 1), in agreement with its basal position in Rubiaceae. Several specimens of *Greenea* included in the analyses could not be identified at species level. We were unable to include any representatives of the genera *Bemsetia* and *Patabea*

as they are only known from their respective type illustrations, and failed in obtaining sequences from extracted samples of *Becheria*, *Charpentiera*, and *Pancheria*. Several taxa were included in the study using accessions from the GenBank database, and we were not always able to obtain complementary material to complete missing data within the data sets (Appendix 1).

DNA EXTRACTION, AMPLIFICATION, SEQUENCING, AND ALIGNMENT

Total DNA was extracted from dried material preserved in silica gel (Chase & Hillis, 1991) or herbarium specimens following the mini-prep procedure of Saghai-Marooof et al. (1984), as modified by Doyle and Doyle (1987). Extracted DNA was cleaned with the QIAquick polymerase chain reaction (PCR) purification kit (Qiagen, Solna, Sweden). PCR reactions were as follows: 27.25 μ l of H₂O, 5 μ l of PCR buffer, 5 μ l of MgCl₂, 5 μ l of 0.1 M tetramethylammonium chloride (TMAc), 4 μ l of dNTP, 0.25 μ l of Taq DNA polymerase (AB-0192 & AB-0192/b; ABGene, Cambridge, U.K.), 0.5 μ l of each primer, and 0.5 μ l of 1% of bovine serum albumin (BSA). PCR amplifications, performed in an Eppendorf Mastercycler (Applied Biosystems, Life Technologies, Carlsbad, California, U.S.A.) gradient, started with an initial melting phase of 2 min. at 95°C, followed by 35 to 37 cycles of 30 sec. at 95°C, 1 min. at 50°C–55°C, and 2 min. at 72°C and ended with a final extension phase of 7 min. at 72°C. In all PCR runs, one reaction was run with water instead of DNA as a negative control to check for contamination.

The *rps16* intron was amplified with the primer pair *rpsF/rpsR2* (Oxelman et al., 1997). For half of the species, we repeatedly failed to obtain amplification in one reaction because of a problematic poly A/T at the 3' end of the intron (Shaw et al., 2005). However, amplification was successful with the internal primer pair *rpsF2/rpsR3* (Bremer et al., 2002), but resulted in sequences 50–70 bp shorter. The entire *trnT-F* region (including the two *trnL* introns) was amplified in two parts. The *trnT-trnL* segment was amplified with the primer pair A1/I (Bremer et al., 2002; Lantz & Bremer, 2004). The second part, *trnL-trnF*, was amplified with primers *c/f* (Taberlet et al., 1991). For *trnL-trnF*, sequencing reactions were performed using the two external primers *c/f* and two internal primers *d/e* (Taberlet et al., 1991) to produce complete sequences of the entire region *trnT-F* with at least partial overlaps (from 20–150 bp). The *rbcL* coding region was amplified in two parts. The first segment was amplified with the primer pair z1/1020R (Andreasen & Bremer, 2000) and the second segment with the couple 3'/427BS (Olmstead et al., 1992;

Andreasen & Bremer, 2000). Sequencing reactions were performed with z1/1020R for the first segment and with 3'/427BS and two internal primers 1204R/895 (Andreasen & Bremer, 2000) for the second part to produce complete sequences of the entire region of *rbcL*, with at least partial overlaps (from 60–90 bp). All sequencing reactions of the markers were performed with Big Dye Terminator v1.1 Cycle Sequencing kit or the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and subsequently analyzed on a 3100 Genetic Analyzer (Applied Biosystems).

The *rps16*, *trnT-F*, and *rbcL* sequences were assembled using the Staden Package version 1.6.0 beta-test (Staden, 1996) or Sequencher 3.1.1 (Gene Codes Corporation, Ann Arbor, Michigan, U.S.A.) and edited manually. Sixty-seven accessions from 33 taxa are new to this paper (Appendix 1). All sequences were aligned manually with Se-Al v1.0al (Rambaut, 1996). The insertion of numerous gaps in nucleotide sequences was required during the alignment procedure for both *trnT-F* and *rps16* (Table 2). Other studies have shown indels to be reliable characters (Lloyd & Calder, 1991; Giribet & Wheeler, 1999; van Dijk et al., 1999; Delarbre et al., 2000; Freudenstein & Chase, 2001; Rouhan et al., 2004). Unambiguous insertions and deletions (indels) were then coded as additional characters by using 0 and 1 symbols for deletions and insertions, respectively (Swofford, 1993).

PHYLOGENETIC ANALYSES

Bayesian analyses were performed with MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001). The Bayesian approach evaluates the posterior probability (PP) of a tree given the character matrix, i.e., the probability that the tree is correct. A partitioned Bayesian analysis was conducted to account for the combination of molecular data with various evolution patterns and standard binary characters of gap coding. For each partition, MrModeltest 2.0 (Nylander, 2004) was used to choose the model of nucleotide substitution that best fit the data, following Akaike's Information Criterion Calculation Method (Akaike, 1974). The selected models were general time reversible (GTR) (Yang, 1994), with among-site substitution rate heterogeneity described by a gamma distribution and all sites constrained to be variable (GTR + G) for *rps16* and the *trnT-L* segment, GTR with a fraction invariant site constraint (GTR + I) for the *trnL-F* segment, and GTR + G + I for *rbcL*. Unambiguous indels were analyzed as an additional partition and treated as binary characters. All analyses were conducted with four independent Markov chains run

Table 2. Indication of the number of accessions included, the length of the aligned matrices, the number of informative characters, and parsimony indices in each data set.

	<i>rps16</i>	<i>trnT-F</i>	<i>rbcL</i>	Combined data
No. of accessions	33	32	29	34
Matrix length	773	1726	1373	3872
Parsimony informative characters	72 (68 substitutions + 4 indels)	148 (119 substitutions + 29 indels)	68 (68 substitutions)	288 (255 substitutions + 33 indels)
Parsimony indices	L – 228; CI – 0.851; RI – 0.883	L – 455; CI – 0.855; RI – 0.888	L – 233; CI – 0.712; RI – 0.785	L – 936; CI – 0.809; RI – 0.851

L, length; CI, consistency index; RI, retention index.

for 2×10^6 Metropolis-coupled Markov chain Monte Carlo (MCMC) generations, with tree sampling every 1×10^3 generations, and burn-in after 500 trees (as detected by plotting the log likelihood scores against generation number). The analyses were run three times using different random starting trees to evaluate the convergence of the likelihood values and posterior clade probabilities (Huelsenbeck et al., 2002). The consensus tree was built using selected trees from each run. Groups characterized by a PP of more than 0.95 were regarded as strongly supported.

To test the topologies inferred from Bayesian analyses, parsimony analyses were also conducted using PAUP* 4.0b8b (Swofford, 2002). The maximum parsimony (MP) trees were found by heuristic search, tree bisection-reconnection (TBR) branch swapping, using 1×10^4 replicates of random stepwise addition, with the MULTREES option on. Characters were given equal weight, gaps were treated as missing data, and only phylogenetically informative indels were coded. The consistency index (CI) (Kluge & Farris, 1969) and the retention index (RI) (Farris, 1989) were calculated to estimate homoplasy. To assess relative support for the identified clades, bootstrap values (BS) (Felsenstein, 1985) were estimated from 1×10^4 replicates, the MULTREES option off, TBR branch swapping, and five random addition sequences. Groups characterized by a bootstrap support of more than 95% were regarded as strongly supported.

To test the null hypothesis that our data sets were homogeneous with respect to phylogenetic information, we used the incongruence length difference test (ILD, also called partition homogeneity test; Farris et al., 1994), as implemented in PAUP*. Invariant sites were removed (Cunningham, 1997) and 1×10^4 replications were performed. The results of the partition homogeneity test indicated that the *rps16*, *trnT-F*, and *rbcL* trees were significantly incongruent ($P = 0.0009$). However, the accuracy of the ILD test tends to be low when the number of the informative

sites is low (Yoder et al., 2001; Darlu & Lecointre, 2002), which is presently observed (Table 2). According to Bremer et al. (2002) and despite incongruence, we favored an analysis of combined data sets.

RESULTS

The results of the analyses conducted in this study are given in the following section, where the sequence characteristics of the individual data sets (Fig. 1, Table 2, unpublished figure for *rbcL*, available from the author for correspondence) are presented, and detailed outputs from the analysis of the combined data sets are given (Fig. 2, Table 2).

SEPARATE ANALYSES

The separate *rps16* and *trnT-F* analyses (Fig. 1A, B), as well as the *rbcL* analysis, all identified the following monophyletic groups: the *Ixora* group (forming seven *Ixora* species, *Hitoea*, *Sideroxyloides*, *Thouarsiora*, *Captaincookia*, *Doricera*, *Myonima*, and *Versteegia*), the *Greenea* group, the *Aleisanthia*–*Aleisanthiopsis* group (not tested for monophyly in the *rbcL* analysis due to the single representative), and Vanguerieae (represented by *Peponidium cystiporon* Byn. ex Cavaco and *Cyclophyllum deplanchei*). Furthermore, *Ixora* was shown to be polyphyletic and *Scyphiphora* left unresolved in a basal position in the Ixoreae alliance. While both the *rps16* and *trnT-F* trees (Fig. 1A, B) identified a highly supported clade containing *Aulacocalyx jasminiflora* Hook. f., *Augusta austrocaledonica* (Brongn.) J. H. Kirkbr., and all sequenced members of the tribes Pavetteae (sensu Andreasen & Bremer, 2000), Octotropideae, Cremasporaeae, Coffeeae, and Albertaeae, this clade was unresolved in the *rbcL* tree (not included). The *Aleisanthia*–*Aleisanthiopsis* group was resolved with high support (BS 99%, PP 1.00) as sister to the *Ixora* group in the *trnT-F* tree (Fig. 1B). This sister-group

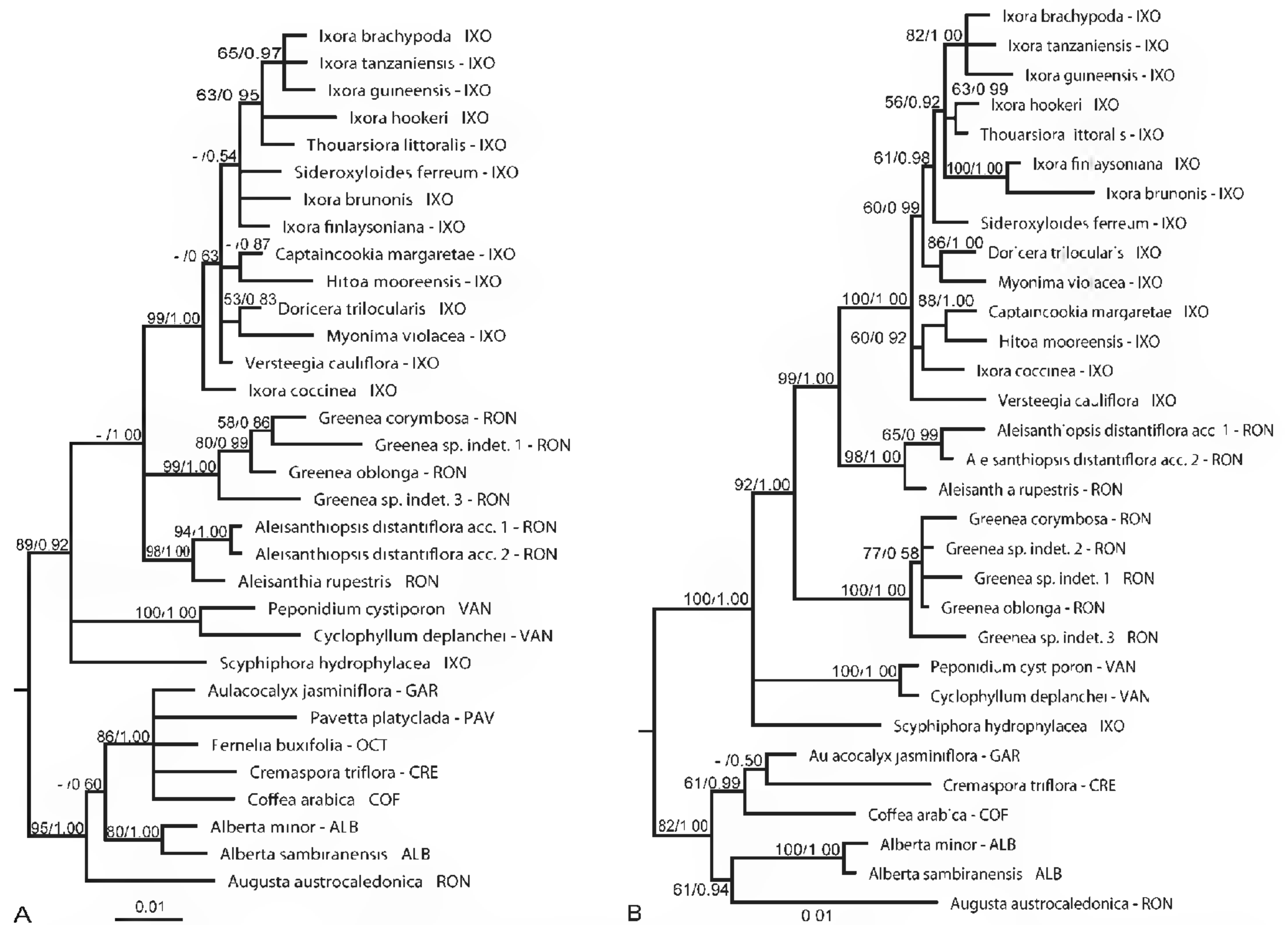


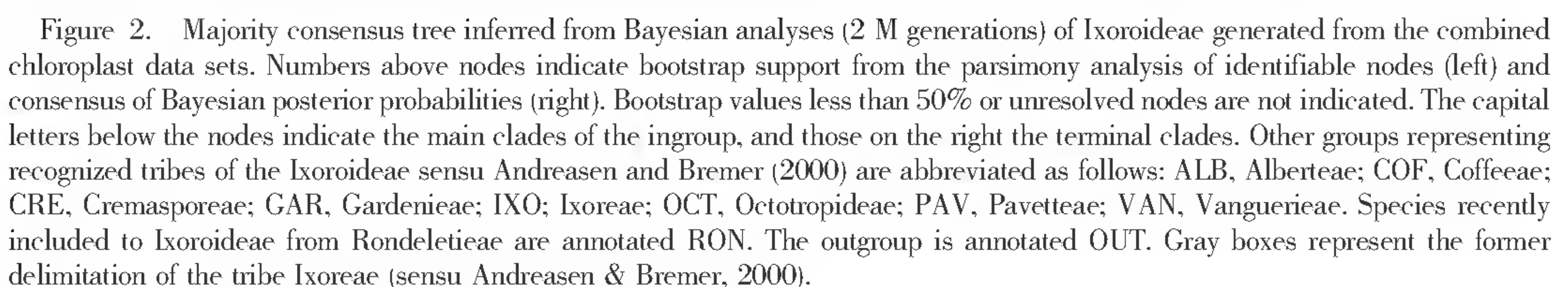
Figure 1. Majority consensus tree inferred from Bayesian analyses of separated markers alignments (2 M generations) of Ixoroideae. —A. Topology generated from the *rps16* data set. —B. Topology generated from the *trnT-F* data set. Numbers above nodes indicate bootstrap support from the parsimony analysis of identifiable nodes (left) and consensus of Bayesian posterior probabilities (right). Bootstrap values less than 50% or unresolved nodes are not indicated. Other groups representing recognized tribes of the Ixoroideae sensu Andreasen and Bremer (2000) are abbreviated as follows: ALB, Albertae; COF, Coffeae; CRE, Cremasporae; GAR, Gardenieae; IXO, Ixoreae; OCT, Octotropideae; PAV, Pavetteae; VAN, Vanguerieae. Species recently included to Ixoroideae from Rondeletieae are annotated RON. Outgroups were removed from trees.

relationship was collapsed in the *rps16* tree (Fig. 1A), while the *Aleisanthia*–*Aleisanthiopsis* group (represented by *Aleisanthiopsis distantifolia* accession 1) and the *Greenea* group were resolved with poor support (BS 55%, PP 0.70) as sister groups in the *rbcL* tree. Within the *Ixora* group, there were some poorly to weakly supported differences between the *rps16*, *trnT-F* (Fig. 1A, B), and *rbcL* trees.

COMBINED ANALYSES

To perform phylogenetic analyses of combined data, we merged the three matrices for all 34 terminal samples (represented in at least two of the three chloroplast DNA [cpDNA] markers) in a combined data set of 3872 sites, including 288 (7.44%) parsimony informative characters. Of the informative characters, 255 (88.54%) were nucleotide substitutions and 33 (11.45%) were indels. In our study, the combined *rps16*–*rbcL*–*trnT-F* tree (Fig. 2) identified

the similar larger monophyletic groups retained in the separate analyses (Fig. 1A, B) with strong support. Internal nodes received high support (Fig. 2). The overall tree topologies of the tree generated from both the parsimony and Bayesian combined analyses were, in general, similar to those of the *rps16* and *trnT-F* trees (Fig. 1A, B). The ingroup taxa were resolved in two main clades, noted as A and B (Fig. 2). Clade A was highly supported (BS 100%, PP 1.00) and contained the *Ixora* group, the *Aleisanthia*–*Aleisanthiopsis* group, the *Greenea* group, exemplar Vanguerieae, and *Scyphiphora hydrophylacea* C. F. Gaertn. (Fig. 2). The relationship between the *Greenea* group (A3) and the *Ixora*–*Aleisanthia*–*Aleisanthiopsis* clade (A1–A2) was strong (BS 100%, PP 1.00). In the combined parsimony analysis, *Scyphiphora* was resolved with poor support (BS less than 50%) as sister to a clade consisting of subclades A1, A2, and A3 (results not shown). Finally, clade B (Fig. 2) received high



support (BS 98%, PP 1.00), while it was not always the case for its internal nodes.

DISCUSSION

First, we compare the sequence characteristics of our *rps16*, *rbcL*, and *trnT-F* data sets with those published for other Rubiaceae groups. Second, we discuss the paraphyly of *Ixora* and the competing circumscriptions of Ixoreae in light of the results of our phylogenetic analyses. Accordingly, a new tribal circumscription of Ixoreae and two new tribes are established.

SEQUENCE CHARACTERISTICS

Our conclusions on the circumscriptions of Ixoreae are based on the combined tree (Fig. 2), as it is the best-supported hypothesis. Most of the informative characters are localized in the *trnT-L* and *trnL-F* spacers rather than the *trnL* introns, consistent with Razafimandimbison and Bremer (2002) and Alejandro et al. (2005). However, many substitutions and most of the indels are within the relatively more conservative *trnL* introns and *rbcL* exon (Table 3); they appear as unambiguous synapomorphies for several clades. The *rbcL* data used in earlier studies about relationships within Rubiaceae are less informative than the *rps16* and *trnT-F* data, consistent with Razafimandimbison and Bremer (2001); *rbcL* sequence data are usually used for assessing interfamilial relationships (Chase et al., 1993; Clegg et al., 1994; Bremer et al., 2002; Shaw et al., 2005).

PARAPHYLY OF *IXORA*

The present analyses demonstrate that *Ixora* as presently delimited is paraphyletic, unless *Captaincookia*, *Doricera*, *Hitoa*, *Myonima*, *Thouarsiora*, and *Versteegia* are all included (Fig. 2). *Ixora coccinea* seems more closely related to *Captaincookia*, *Hitoa*, and *Versteegia* than it is to the six sequenced species of *Ixora*. Plus, *Doricera* and *Myonima* appear more closely related to the six sampled *Ixora* species than they are to *I. coccinea*. The inclusion of *Hitoa*, *Sideroxyloides*, and *Thouarsiora* with *Ixora* is supported by our studies. On the other hand, very few distinctive morphological features distinguish *Ixora* from its allied genera, such as hypoc crateriform versus infundibuliform corollas and soft versus bony fruits (Bridson & Robbrecht, 1985). With respect to *Ixora*, problematic generic circumscriptions were already observed in Rubiaceae, especially among large genera (e.g., *Psychotria* L., *Galium* L., *Oldenlandia* L.). Phylogenetic analyses based on multiple nuclear

and chloroplast markers and a much larger sampling of *Ixora* and affined taxa are being undertaken to specifically address the generic limits of *Ixora*.

CIRCUMSCRIPTION OF IXOREAE

The results of the present study do not support the two recently suggested circumscriptions of Ixoreae. Ixoreae sensu Andreassen and Bremer (2000) is not monophyletic, unless *Scyphiphora* is excluded and *Captaincookia*, *Doricera*, *Myonima*, and *Versteegia* are included. Ixoreae sensu Robbrecht and Manen (2006) is polyphyletic, as it includes *Scyphiphora* and does not comprise both *Captaincookia* and *Doricera*. Our analyses further corroborate the exclusion of the monotypic *Captaincookia* and *Doricera* from Pavetteae sensu Andreassen and Bremer (2000), represented here by *Pavetta platyclada* K. Schum. & Lauterb., and support the inclusion of the former two genera in a broadly circumscribed Ixoreae. The phylogenetic position of *Aleisanthia*, *Aleisanthiopsis*, and *Greenea* within the Ixoroideae, as revealed by Rova et al. (2002), and its close relationship with *Ixora* s.l., is also corroborated by our results (Fig. 2).

The phylogeny (Fig. 2) clearly shows that Ixoreae needs a new circumscription. Whether Ixoreae should be recognized in a narrow sense (i.e., including only *Ixora* s.l.) or in a broad sense (i.e., *Ixora* s.l. plus *Aleisanthia*, *Aleisanthiopsis*, and *Greenea*) is a matter of taxonomic judgment. Recognizing Ixoreae in a narrow sense would force us to raise both the *Aleisanthia*–*Aleisanthiopsis* and *Greenea* groups to the tribal level. This would make Ixoreae s. str. homogeneous morphologically (Table 4) but cause nomenclatural novelties. In contrast, one may propose a broad circumscription of Ixoreae (including genera of subclades A1 to A3 in Fig. 2), which requires no nomenclatural changes but perhaps necessitates descriptions of three new subtribal taxa. However, merging *Aleisanthia*, *Aleisanthiopsis*, and *Greenea* in Ixoreae would make the tribe morphologically heterogeneous (Table 4) and diagnosable only by its aestivation contorted to the left (Robbrecht & Manen, 2006) and its lack of raphides, two features commonly found throughout the Ixoroideae. We favor a narrow and well-defined circumscription of Ixoreae, which contains the following eight genera of subclade A1 (Fig. 2): *Captaincookia*, *Doricera*, *Hitoa*, *Ixora* (as polyphyletic), *Myonima*, *Sideroxyloides*, *Thouarsiora*, and *Versteegia*. Accordingly, we recognize both the distinctive *Aleisanthia*–*Aleisanthiopsis* (subclade A2) and *Greenea* (subclade A3) at the tribal level.

Ixoreae s. str. (e.g., subclade A1, Fig. 2) can easily be diagnosed by its articulated petioles, 4-merous flowers, corolla contorted to the left in bud, exerted

Table 3. Unambiguous molecular synapomorphies for recognized tribal and generic lineages.

Lineages	No. of synapomorphic substitutions	Synapomorphic insertions	Synapomorphic deletions
Ixoreae	<i>trnT-L</i> (spacer): 1 <i>trnL-F</i> (introns): 6 <i>rbcL</i> (exon): 3	<i>rps16</i> (intron): CTAAA	<i>trnT-L</i> (spacer): T
Aleisanthieae	<i>trnL-F</i> (intron): 2	<i>trnT-L</i> (spacer): CATAATCATATATTTCTA <i>trnL-F</i> (introns): CTTTTAATTG	
Greeneeae	<i>trnT-L</i> (spacer): 2 <i>trnL-F</i> (introns): 5 <i>rps16</i> (intron): 4 <i>rbcL</i> (exon): 3	<i>rps16</i> (intron): T	<i>trnT-L</i> (spacer): GTATA <i>trnL-F</i> (introns): CAAAA <i>rps16</i> (intron): TTTTAT
<i>Scyphiphora</i>	<i>trnT-L</i> (spacer): 7 <i>trnL-F</i> (introns): 10 <i>rps16</i> (intron): 5	<i>trnT-L</i> (spacer): AAATA; T; T; TTTT <i>trnL-F</i> (introns): GAAAATAT; T	<i>trnT-L</i> (spacer): A; A <i>trnL-F</i> (introns): TTAATGA; ATTCAATTTAT <i>rps16</i> (intron): T

stamens, drupaceous fruits and uniovular locules, sclerified endocarp, and entire endosperm (Bremekamp, 1934, 1937; De Block, 1997; Andreasen & Bremer, 2000; Table 4). The *Aleisanthia*–*Aleisanthiopsis* group (as “Aleisanthieae”; subclade A2, Fig. 2) is a well-defined clade that can be diagnosed by the woolly hairs on the abaxial leaf surface, scorpioid inflorescences, infundibuliform corollas, inserted stamens, multiovulate ovaries, and capsular fruits (Tange, 1996a, b; Table 4). Similarly, *Greenea* (as “Greeneeae”; subclade A3, Fig. 2) can be distinguished from Ixoreae and the *Aleisanthia*–*Aleisanthiopsis* group by protogyny, flowers without an obvious calyx tube, simply consisting of minute triangular free lobes (Puff et al., 2005; Table 4) and the lack of secondary pollen presentation (Tange, 1996b).

SCYPHIPHORA

We find no support for the phylogenetic placement of *Scyphiphora* as sister to *Ixora* s. str. shown by Andreasen and Bremer (2000). Its position in Ixoreae sensu Robbrecht and Manen (2006) is not corroborated by our results, as it is placed in an unresolved trichotomy with Ixoreae s.l. and Vanguerieae within clade A (Fig. 2). For now, *Scyphiphora* should be considered to be unplaced but close to Ixoreae, Aleisanthieae, Greeneeae, and Vanguerieae. Few distinct morphological features separate *Scyphiphora* from the taxa in clade A (Fig. 2, Table 4). The presence of two ovules per locule (Baillon, 1880; Puff & Rohrhofer, 1993) in *Scyphiphora* is a rather unusual feature within this clade A, as well as within Rubiaceae. Furthermore, fibrous and very thick pyrenes are also rare and probably represent an adaptation to sea dispersal, as *S. hydrophylacea* is

restricted to mangrove habitats. The genus has contorted aestivation like Ixoreae (as here delimited), Aleisanthieae, and Greeneeae, whereas Vanguerieae representatives (subclade A4, Fig. 2) have valvate aestivation. Conversely, *Scyphiphora* resembles Vanguerieae (and *Aleisanthia*) in its axillary inflorescences. Despite its supported position within clade A (Fig. 2), *Scyphiphora* remains both unresolved from molecular data and ambiguous in its putative affinities within clade A tribes according to morphology.

TAXONOMIC SYNOPSIS

The tribal concept of Ixoreae is better understood with results from molecular phylogenetic analyses. Bentham (1849) used the name Ixoreae first but only provisionally (Darwin, 1976: 603). The current use of the name correctly attributes Ixoreae to Gray (1858). The following treatment presents an emended description of Ixoreae and recognition of two new tribes (for *Aleisanthia*–*Aleisanthiopsis* and for *Greenea*) for which no names are available.

- 1. Ixoreae** A. Gray., Proc. Amer. Acad. Arts 4: 39. 1858, non Ixoreae Griseb., nom. illeg., p.p., Fl. Brit. India 1: 337. 1861, as subtribe [including *Coffea*], nec Ixoreae Hook. f., nom. illeg., p.p., Gen. Pl., 2: 9, 22. 1873, as tribe [including *Coffea*], nec Ixoreae K. Schum., nom. illeg., p.p., Nat. Pflanzenfam. 4: 1891; as tribe [including *Coffea* and *Pavetta*]. TYPE: *Ixora* L.

Coffeeae DC. p.p., Ann. Mus. Natl. Hist. Nat. 9: 217. 1807, as tribe.

Pavetteae A. Rich. ex Dumort. p.p., Anal. Fam. Pl.: 33. 1829, as tribe.

Table 4. Summary of selected morphological characters relevant to Ixoreae alliance as presently circumscribed. Unambiguous morphological features that characterize the recognized tribes and *Scyphiphora* within the main clade A of Figure 2 are in boldface.

	Ixoreae	Aleisanthieae	Greeneeae	Vanguerieae	<i>Scyphiphora</i>
Woolly hairs	absent	present	absent	absent	absent
Petioles	articulate	not articulate	not articulate	not articulate	articulate
Domatia	absent	present	present	present	absent
Inflorescence position	terminal/cauliflorous	terminal/axillary	terminal	axillary	axillary
Inflorescence type	cymose	scorpioid	scorpioid	cymose	cymose
Flowers	4-merous	5-merous	5-merous	4- to 5-merous	4-merous
Corolla aestivation	contorted	contorted	contorted	valvate	contorted
Calyx tube	present	present	absent	present	present
Stamens	exserted	inserted	inserted	inserted/exserted	exserted
Ovule numbers	1-ovule/carpel	many ovules/carpel	many ovules/carpel	1-ovule/carpel	2-ovules/carpel
Fruits	drupaceous	capsular	capsular	drupaceous	drupaceous
Sclerified endocarps	present	absent	absent	present	present
Dispersal mode	zoochorous	anemochorous	anemochorous	zoochorous	sea currents
Secondary pollen presentation	present	present	absent	present	present
Pollen grains	colporate	pororate/colporate	colporate	po(ro)rate	colporate
Sexuality	protandrous	protandrous	protogynous	protandrous	protandrous

Ixoreae representatives are easily distinguishable by the following combination of characters: leaf petiole articulate; inflorescences as small to large cymes, terminal (sometimes on brachyblasts or cauliflorous); flowers 4-merous, protandrous; aestivation contorted to the left; corolla hypocrateriform (*Captaincookia* excepted); stamens exserted; secondary pollen presentation; ovary 2(to 7)-locular; locule uniovulate; fruit drupaceous, 2(to 7)-locular; pyrene leathery to crustaceous; seed with circular adaxial excavation extending into a basal groove; endosperm entire.

Included genera: *Becheria*, *Bemsetia*, *Captaincookia*, *Doricera*, *Hitoa*, *Ixora*, *Myonima*, *Patabea*, *Sideroxyloides*, *Thouarsiora*, *Versteegia*.

Ixoreae (subclade A1, Fig. 2) as presently circumscribed is supported by several unambiguous synapomorphies among three chloroplast markers (e.g., Table 3) and a homogeneous morphology. The following genera are currently recognized as *Ixora* synonyms: *Becheria*, *Bemsetia*, *Hitoa*, *Patabea*, *Sideroxyloides*, *Thouarsiora*, but the paraphyly of *Ixora* questions generic circumscriptions within Ixoreae.

2. Aleisanthieae Mouly, J. Florence & B. Bremer, tribus nov. TYPE: *Aleisanthia* Ridl.

Tribus nova quae ab Ixoreis A. Gray lamina foliari subtus plerumque omnino lanosa, inflorescentia scorpioidea, floribus pentameris atque ovarii loculis multiovulatis praecipue differt.

Aleisanthieae is distinguished by the following combination of characters: blades covered with woolly

hairs on the abaxial leaf surface; inflorescence scorpioid, axillary or terminal; flowers 5-merous, small, protandrous; calyx lobes small and triangular to rounded; aestivation contorted to the left; corolla infundibuliform; stamens included; secondary pollen presentation; ovary 2-locular, multiovulate; fruit capsular.

Included genera: *Aleisanthia*, *Aleisanthiopsis*, *Greeniopsis* Merr.

The Aleisanthieae clade comprises the two South-east Asian genera *Aleisanthia* and *Aleisanthiopsis* and is resolved as sister to the pantropical Ixoreae s. str. Aleisanthieae is well supported by unambiguous molecular evidence, with synapomorphic substitutions and indels (Table 3). The woolly hairs on the abaxial leaf surface (Table 4) represent a rare feature in Ixoroideae and may be regarded as a synapomorphy of the Aleisanthieae. Tange (1996b) also associated the Philippine genus *Greeniopsis* (Merrill, 1909), with seven species, with *Aleisanthia* and *Aleisanthiopsis*, but reported no presence of woolly hairs on the leaves. However, *G. discolor* Merr. does possess these typical hairs (Mouly, pers. obs.). In the genera *Aleisanthia* and *Greeniopsis*, styler complexes are observed, similar to those described in Vanguerieae (Igersheim, 1993; Lantz et al., 2002), but these were not observed in *Aleisanthiopsis distantiflora* (Merr.) Tange (Tange, 1996b). The pollen grains are 3-pororate in *Aleisanthia* and *Greeniopsis*, although the pollen stains in *Aleisanthiopsis* are 3-colporate. Inflorescences are axillary in *Aleisanthia* but are terminal in *Aleisanthiopsis* and *Greeniopsis*. Our tentative placement of *Greeniopsis* in

Aleisanthieae based on morphological resemblance needs to be confirmed from molecular data.

3. Greeneeae Mouly, J. Florence & B. Bremer, tribus nov. TYPE: *Greenea* Wight & Arn.

Tribus nova quae ab Aleisanthieis floribus proterogynis, calyce sine tubo manifesto atque stigmatibus linearibus sine pollinis praesentatione secundaria praecipue differt.

The tribe Greeneeae is characterized by the following combination of characters: leaves without abaxial woolly hairs (but rarely glabrous); inflorescence terminal, scorpioid; flowers 5-merous, small, protogynous; calyx without obvious tube; aestivation contorted to the left; corolla infundibuliform to campanulate; stamens included; primary pollen presentation; ovary 2-locular, multiovulate; fruit capsular.

Included genera: *Greenea*, *Spathichlamys* R. Parker.

The absence of secondary pollen presentation in *Greenea* (Tange, 1996b) is uncommon within the studied lineage (clade A, Fig. 2). Furthermore, the protogyny of the *Greenea* species is also unique: the stigmatic lobes are partially to completely exerted and opened at the latest bud stage, while anthers are accessible to pollinators only after opening and enlargement of the corolla tube. Otherwise, in all species with secondary pollen presentation, an initial functionally male stage precedes the functionally female stage during anthesis (Nilsson et al., 1990; Puff et al., 1996). The disappearance of secondary pollen presentation might be linked to the adaptation to protogyny. Greeneeae is supported by many unambiguous molecular synapomorphies (see Table 3). The species *G. commersonii* (Korth.) Boerl. (Boerlage, 1891), previously described in the genus *Rhombospora* Korth. (Korthals, 1850), also shares the diagnostic morphological features of *Greenea*, but was not investigated. The monotypic Indonesian genus *Spathichlamys* (Parker, 1931; Ridsdale, 1982; Tange, 1996b) is identical morphologically to *Greenea* from which it differs only by the corolla that splits and rolls up at anthesis (Ridsdale, 1982).

CONCLUSIONS

The present study clearly shows that *Ixora* as currently delimited is paraphyletic and Ixoreae sensu Andreassen and Bremer (2000) and sensu Robbrecht and Manen (2006) are non-monophyletic. The monophyly of a broadly circumscribed and morphologically heterogeneous Ixoreae alliance (including *Ixora*, *Captaincookia*, *Doricera*, *Hitoa*, *Myonima*, *Sideroxyloides*, *Thouarsiora*, *Versteegia*, plus *Aleisanthia*, *Aleisanthiopsis*, and *Greenea*) is strongly supported. In order to recognize monophyletic and morphologi-

cally consistent groups, we adopt a narrowly circumscribed Ixoreae (including *Becheria*, *Bemsetia*, *Captaincookia*, *Doricera*, *Hitoa*, the paraphyletic *Ixora*, *Myonima*, *Patabea*, *Sideroxyloides*, *Thouarsiora*, and *Versteegia*) and erect the *Aleisanthia*–*Aleisanthiopsis* and *Greenea* groups at tribal level, Aleisanthieae and Greeneeae, respectively. Finally, the Malesian Aleisanthieae and the pantropical Ixoreae s. str. are sister groups and the Southeast Asian Greeneeae is sister to the Ixoreae–Aleisanthieae clade.

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Appendix 1. Specimen vouchers, origin, and accession numbers of *rps16*, *trnT-F*, and *rbcL* sequences used in molecular analyses. In the Voucher/Reference column, indications follow the appearance of genic regions when multiple, or refer to all genic regions when single. Herbarium acronyms are listed in parentheses after voucher information. Newly published sequences are annotated with an asterisk after GenBank accessions.

Species	Vouchers/References	Origins	Accession numbers <i>rps16</i>	Accession numbers <i>trnT-F</i>	Accession numbers <i>rbcL</i>
Outgroup					
<i>Luculia gratissima</i> (Wall.) Sweet	<i>CT 80064</i> (UPS)	unknown	EU817448*	EU817472*	EU817429*
Alberteae					
<i>Alberta minor</i> Baill.	<i>Razafimandimbison S. G. 558</i> (UPS)	Madagascar	EF205637	EU817452*	EU817410*
<i>A. sambiranensis</i> Homolle ex Cavaco	<i>Razafimandimbison S. G. 360</i> (UPS)	Madagascar	EF205645	EU817474*	EU817431*
Cremasporeae					
<i>Cremaspora triflora</i> K. Schum.	Persson, 2000; Persson, 2000; Andreassen & Bremer, 1996	unknown	AF200990	AF201040	Z68856
Coffeeae					
<i>Coffea arabica</i> L.	Andersson & Rova, 1999; Struwe et al., 1998; Bremer et al., 1995	unknown	AF004038	AF102405	X83631
Gardenieae					
<i>Aulacocalyx jasminiflora</i> Hook. f.	<i>Schmidt H. H. et al. 1672</i> (MO)	Ghana	EF205639	EU817455*	EU817413*
Ixoreae					
<i>Captaincookia margaretae</i> N. Hallé	<i>Mouly A. & Innocente E. 267</i> (P)	New Caledonia	EU817436*	EU817456*	EU817415*
<i>Doricera trilocularis</i> (Balf. f.) Verdc.	<i>Lesouef J. Y. 31</i> (TAN)	Mascarenes	EU817437*	EU817457*	EU817417*
<i>Hitoea mooreensis</i> Nadeaud	<i>Florence J. s.n.</i> (P)	French Polynesia	EU817441*	EU817462*	EU817420*
<i>Ixora brachypoda</i> DC.	<i>Bradley A. F., et al. 1022</i> (MO)	Gabon	EU817442*	EU817463*	EU817421*
<i>I. brunonis</i> Wall. ex G. Don	<i>Larsen K. et al. 43463</i> (P)	Thailand	EU817446*	EU817470*	EU817427*
<i>I. coccinea</i> L.	<i>Bremer B. 2719</i> (UPS); <i>Bremer B. 2719</i> (UPS); <i>Bremer B. 3104</i> (UPS)	cultivated in Stockholm, Sweden	EF205641	EU817464*	X83646
<i>I. finlaysoniana</i> Wall. ex G. Don	<i>Luke Q. 9042</i> (UPS)	Kenya	EF205643	EU817466*	EU817423*
<i>I. guineensis</i> Benth.	<i>Gereau R. E. et al. 5601</i> (MO)	Ghana	EU817443*	EU817467*	EU817424*
<i>I. hookeri</i> (Oudem.) Bremek.	<i>Mouly A. & Florence J. 342</i> (P)	French Polynesia	EU817444*	EU817468*	EU817425*
<i>I. tanzaniensis</i> Bridson	<i>Luke Q. 9304</i> (S)	Tanzania	EU817447*	EU817471*	EU817428*
<i>Myonima violacea</i> (Lam.) Verdc.	<i>Lorence D. H. 1526</i> (P)	Mascarenes	EU817449*	EU817473*	EU817430*
<i>Scyphiphora hydrophylacea</i> C. F. Gaertn.	<i>Bremer K. et al. 95</i> (S)	Sri Lanka	EU817450*	EU817475*	EU817432*
<i>Sideroxyloides ferreum</i> Jacq.	<i>Taylor C. 11693</i> (MO)	Caribbean	EF205642	EU817465*	EU817422*
<i>Thouarsiora littoralis</i> Homolle ex Arènes	<i>McPherson G. & Rabenantoandro J. 18287</i> (MO)	Madagascar	EU817445*	EU817469*	EU817426*

Appendix 1. Continued.

Species	Vouchers/References	Origins	Accession numbers <i>rps16</i>	Accession numbers <i>trnT-F</i>	Accession numbers <i>rbcL</i>
<i>Versteegia cauliflora</i> Valetou	<i>Drodz P. & Molem K. s.n. (UPS)</i>	cultivated in Bogor, Indonesia	EU817451*	EU817476*	EU817433*
Octotropideae					
<i>Fernelia buxifolia</i> C. F. Gaertn.	Rova, unpubl.; –; Andreassen & Bremer, 2000	unknown	AF244892	–	AJ286704
Pavetteae					
<i>Pavetta platyclada</i> K. Schum. & Lauterb.	<i>Drozd P. & Molem K. s.n. (UPS)</i>	New Guinea	AJ320082	–	AJ318451
Rondeletieae					
<i>Aleisanthia rupestris</i> (Ridl.) Ridl.	<i>Tange C. 45171 (AAU)</i>	Malaysia	AF242902	AF152660	–
<i>Aleisanthiopsis distantiflora</i> (Merr.) Tange	accession 1: <i>Kessler P. J. A. et al. 41 (P)</i>	Borneo	EU817434*	EU817453*	EU817411*
<i>A. distantiflora</i>	accession 2: <i>Tange C. 46977 (AAU)</i>	Borneo	AF242903	AF152658	–
<i>Augusta austrocaledonica</i> (Brongn.) J. H. Kirkbr.	<i>Mouly A. & Innocente E. 237 (P)</i>	New Caledonia	EF205638	EU817454*	EU817412*
<i>Greenea corymbosa</i> (Jack) Voigt	<i>Larsen K. et al. 44102 (AAU)</i>	Thailand	AF242961	AF152657	–
<i>G. oblonga</i> Craib	<i>Larsen K. & Larsen S. S. 33451 (P)</i>	Thailand	EU817459*	EU817439*	–
<i>Greenea</i> sp. indet. 1	<i>Larsen K. & Larsen S. S. 33378 (P)</i>	Thailand	EU817440*	EU817461*	EU817419*
<i>Greenea</i> sp. indet. 2	<i>Larsen K. et al. 43140 (P)</i>	Thailand	–	EU817460*	EU817418*
<i>Greenea</i> sp. indet. 3	<i>Beusekom C. F. van et al. 752 (P)</i>	Thailand	EU817438*	EU817458*	–
Vanguerieae					
<i>Cyclophyllum deplanchei</i> Hook. f.	<i>Mouly A. & Innocente E. 228 (P)</i>	New Caledonia	EF205640	EF205631	EU817416*
<i>Peponidium cystiporon</i> (Byn. ex Cavaco) Razafim., Lantz & B. Bremer	<i>Labat J.-N. et al. 3280 (P)</i>	Comores	EU817435*	EF205629	EU817414*